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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of
Kenji TANAKA et al.
Serial No. 60/272,981
Filed March 2, 2001
For : PROTEIN CHIP FOR PROTEOME ANALYSIS, PREPARATION METHOD THEREOF AND NOVEL PROTEOME ANALYSIS METHOD USING THE SAME

Group Art Unit
Examiner

TRANSLATOR'S DECLARATION

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I, Ritsuko Arimura, declare:

That I am well acquainted with both the Japanese and English languages;

That the attached document represents a true English translation of US Patent Application Serial No. 60/272,981 filed on March 2, 2001; and

That I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 16th day of October, 2001.

Ritsuko Arimura

SPECIFICATION

PROTEIN CHIP FOR PROTEOME ANALYSIS, PREPARATION METHOD THEREOF AND NOVEL PROTEOME ANALYSIS METHOD USING THE SAME

TECHNICAL FIELD OF THE INVENTION

The present invention relates to a novel method and tools therefor for proteome analysis, which are applicable to simultaneous screening and quantitation of membrane proteins and ligands thereof, as well as to the analysis of interaction between them. More particularly, the present invention relates to a method for preparing a protein chip for quick and facilitated proteome analysis, a protein chip for proteome analysis obtained by this method, and to a novel method for proteome analysis using this protein chip.

BACKGROUND OF THE INVENTION

With the advance in the fundamental researches for drug discovery, including molecular biology and genomics (genome science), the landscape of drug discovery in these several years has been rapidly changing toward new prospects represented by genomic drug discovery. The discovery of a novel medicine rests on the finding of a substance that has a unique physiological activity in certain disease(s). The substances that are responsible for such physiological activities are mostly proteins, and elucidation of the structure and function of proteins is the essential problem in the development of medicines.

Because the function (physiological action) of a protein cannot be predicted from the base sequence determined by genomics, establishment of a method for connecting the genetic information to a new drug is demanded for post-genomics. One of them drawing attention is proteomics (protein science). There are 140000 species of proteins to be translated. The isolation·identification and deduction of the function of all of these is the goal of the proteomics. As the situation stands, how to connect the genomic information to the

understanding of protein functions is the strategic goal of the drug discovery in the 21st century.

For biogenic activities from fertilization to development, differentiation, growth, metabolism and to death, proteins embedded in membranes carry out important functions. These membrane proteins are present as a membrane receptor to transmit extracellular information to the inside of the cell, as a specific membrane transporter of physiological substances from the inside to the outside of the cell and vice versa, and as lining proteins of membranes that support dynamic membrane structures. These membrane-associated proteins are difficult to purify or isolate, and impose many difficulties in functional analyses, which delayed the researches.

The relationship between structure and molecular activity is a fundamental item in the study of biology. The structure-activity relationship is critical for the understanding of any biological reaction, such as enzyme functions, method of intercellular communication, and cellular regulation and feedback system.

Certain species of macromolecules are known to interact and bind with other molecules, having highly specific 3-dimensional and electron distribution. Any macromolecule having such specificity is considered to be a receptor, whether it is an enzyme that catalyzes hydrolysis of metabolic intermediates, a cell surface protein that mediates membrane transport of ions, a glycoprotein useful for identifying a particular cell from neighboring cells, an IgG-class antibody circulating in plasma, an oligonucleotide sequence of nuclear DNA or something else. Various molecules that a receptor selectively binds with are known as ligands.

Many methods are available to discover unknown receptors and ligands, but the information obtainable from conventional experiments is sometimes limited by the number and type of available ligands. Discovery of a complex type receptor

consisting of plural peptides is associated with still more difficult problems. Novel receptors and ligands are found by new methods, such as X-ray crystal diffraction or genetic recombination techniques. However, such new methods depend on accidental coincidence and take a long period of biochemical research, or are applicable to extremely limited molecular species.

The high diversity of protein molecular structures and functions involved in the biogenic activities in general is incomparable with the diversity of DNA molecules. In this sense, trying the strategy of genomics, which achieved a success only by applying the DNA sequencing method to structurally similar 24 human chromosomes, directly on proteomics that deals with the objects affluent in diversity is impractical. Only with the DNA sequence, prediction of biogenic activity is impossible. Thus, proteomics capable of elucidation of protein functions is awaited.

Given the consideration set out in the above, a study of membrane receptor and membrane transporter as the targets of proteomics for the elucidation of a part of the physiological function thereof (=identification of physiological ligand) is of greater significance.

Conventional study of proteomics has exclusively relied on the two-dimensional electrophoresis method as a means of separating proteins. The protein separated by two-dimensional electrophoresis is detected by segmenting gel into small fragments, extracting the protein from each fragment using a particular solution, and analyzing each extract solution by mass spectrometer. Complicated manipulations of this method refuse miniaturization of a device, shortening of measurement time, processing of multiple samples, or automation of entire device, thereby prevent realization of quick and convenient proteome analysis.

There are ongoing developments of techniques for direct

analysis of protein from biological sample without separation of proteins, based on differential display using a protein chip. A system called SELDI-Based ProteinChip® System (Ciphergen Biosystems Inc.) that uses a metallic protein chip and a time-of-flight mass spectrometer (TOF-MS) in combination is one of them. ProteinChip® can sort and capture proteins on the chip surface according to the chemical properties of the protein, because each spot regularly arranged on the chip surface bears property of, for example, normal phase, reversed phase, hydrophilicity, hydrophobicity, cation exchangeability, anion exchangeability, IMAC and the like. ProteinChip® is directly applied to TOF-MS, which in turn affords information of the molecular weight of the captured protein. Inasmuch as this method captures protein on a chip surface using chemical affinity, protein without the affinity for any binding group on the chip surface is not captured but washed away. This means the obtainable information does not cover all the proteins in a biological sample.

BRIEF SUMMARY OF THE INVENTION

The present invention provides a novel method for proteome analysis based on the basic idea of grouping all the proteins into membrane-associated proteins and soluble proteins, which method comprises; preparing respectively membrane proteins in the presence of a lipid bilayer and their physiological ligands as soluble proteins with their physiological actions maintained; simultaneously isolating and purifying the both utilizing the biological affinity of the both; and elucidating the functions of them (overall concept of the present invention is shown in Fig. 1). Particularly, the present invention provides a method of separating and capturing soluble proteins (step A in Fig. 1), which is the fundamental technique of the proteome analysis, and a method of detecting the captured protein which is the fundamental technique of the proteome analysis of the invention (step D in

Fig. 1).

More particularly, the present invention provides; a method for preparing a protein chip for proteome analysis, which method comprises separating only soluble proteins containing ligands from a biological sample by a two-dimensional electrophoresis method, and transferring the soluble protein directly onto a protein chip from the gel of the electrophoresis and; a method for proteomes analysis, which method comprises detecting the soluble proteins that are transferred onto the protein chip by mass spectrometric analysis. Because the series of these methods do not require, unlike conventional methods, fragmentation of gel, extraction of protein from the gel fragments or mass spectrometric analysis of each extract solution, the analysis time can be shortened, the space can be saved and the device can be automated, thereby enabling large scale analyses.

The present invention further provides a novel method for proteome analysis, which method comprises detecting membrane protein-ligand complexes on protein chips at a time by combining the above-mentioned method with step B for attaching or penetrating a membrane protein to or through a synthetic liposomal membrane and further with step C wherein a liposome having a membrane protein transferred is brought into contact with a protein chip on which ligands (soluble proteins) have been deposited and a membrane protein-ligand complex is isolated utilizing their biological affinity, and integrating the obtained data and creating database.

The elucidation of membrane proteins and physiological ligands thereof offers an innovative screening system for the development of a novel therapeutic agent for various diseases. Moreover, the database of disease-related membrane proteins and ligands, inclusive of novel and known ones, enables dynamic molecular level elucidation of diseases that the genomic pharmacology cannot achieve, and expected to lead to

the development of novel diagnostic methods and novel therapeutic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the overall concept and respective constituent elements of the present invention.

Fig. 2 is one embodiment of the protein chip used for the present invention.

Fig. 3 is one embodiment of the ligand-receptor (membrane protein) matrix table used in the present invention.

Fig. 4 shows a photograph of a protein chip on which urokinase has been transferred and coomasie-brilliant-blue-stained polyacrylamide gel before and after the transfer to the protein chip.

Fig. 5 shows the results of mass spectrometric analysis of urokinase on the protein chip.

Fig. 6 shows the results of quantitative analysis of urokinase transferred on the protein chip.

DETAILED DESCRIPTION OF THE INVENTION

1. Preparation of protein chip for proteome analysis

The first step for the preparation of the protein chip for proteome analysis of the present invention is separation of soluble protein by two-dimensional gel electrophoresis.

The two-dimensional electrophoresis can be performed using a commercially available product, or an originally devised apparatus. The one dimensional migration is based on the separation by the isoelectric point of the protein, and the two-dimensional migration is based on the separation according to the molecular weight of the protein. The size of the gel used for electrophoresis is not subject to any particular limitation. While 10 cm x10 cm is typical, but 20 cm x 20 cm or other sizes can be used where necessary. While the basic material of the gel is polyacrylamide, a different medium such as agarose gel, cellulose acetate membrane and the like can be also used depending on the purpose. The gel

concentration may be constant or may have a gradient.

The second step for the preparation of the protein chip for proteome analysis of the present invention is transfer of the protein migrated and spread in the gel onto a protein chip.

By the "protein chip" is meant a medium to which soluble molecules generally called ligands are bound. It means a medium having a form that can be adapted automatically and instantaneously to a high sensitivity analytical instrument, after separating a ligands by a high precision analytical method such as two-dimensional electrophoresis or high performance liquid chromatography and the like. In most embodiments, the shape of the medium is substantially planar but in some embodiments, it is in the form of particle, strand, precipitate, gel, sheet, tube, spherical body, container, capillary, pad, slice, film, plate, slide and other various surface structures. Essentially, an optional convenient form can be employed in the present invention. The surface of the medium may be biological, non-biological, organic or inorganic, or a combination of these, and is formed on the surface of a hard support on which the reaction described in the present specification occurs. The surface of the medium is selected to afford suitable protein adsorbing property. Examples thereof include functionalized glass, Si, Ge, GaAs, GaP, SiO_2, SiN_4, reforming silicone, a wide range of gels and polymers, such as (poly)tetrafluoroethylene, (poly)vinylidene difluoride, polystyrene, polycarbonate, or a combination of these and the like. Examples of the surface of the medium having plural monomer or polymer sequences include linear or circular polymers of nucleic acid, polysaccharide, lipid, peptide having α-, β- or ω-amino acid; gel surface carrier used for chromatography (anionic/cationic compounds, hydrophobic compound consisting of 1 to 18 carbon chains, carrier crosslinked with hydrophilic compound, such as silica, nitrocellulose, cellulose acetate, agarose and the like,

etc.); synthetic homopolymers such as polyurethane, polyester, polycarbonate, polyurea, polyamide, polyethyleneimine, polyallylenesulfide, polysiloxane, polyimide, polyacetate and the like; hetetopolymers that are conjugates of any of the above-mentioned compound and a known drug or natural compound bound thereto (covalent or non-covalent bond); and other polymers that will be found suited after general view of this disclosure.

In a preferable embodiment of the present invention, the shape of the protein chip is devised to completely match the sample inlet of the mass spectrometer to be used thereafter. For example, a "cluster protein chip" is mentioned, which has split lines previously made to allow easy separation of each one of the clusters, which fits the sample inlet of the mass spectrometer, after transfer of a protein to a collective type protein chip fitting the size of a gel after electrophoresis. Fig. 2 shows one embodiment of a preferable protein chip of the present invention.

In conventional mass spectrometry, samples are measured one by one, or only limited samples are measured at a time, because of one dimensional transfer of the protein chip, like the aforementioned cluster protein chip. In contrast, the completely automated proteomics analysis according to the present invention enables analysis of all the proteins spread two-dimensionally by electrophoresis by moving the laser nozzle or a table carrying the protein chip in the two-dimensional directions for continuous whole scanning (intermittent scanning leaves proteins free of laser beam irradiation, namely unanalyzed proteins). This method combined with intermittent scanning makes it possible to mount 96 kinds of samples dispensed to a 96-well plate altogether on a protein chip (96 kinds of samples are arranged at the same intervals on a rectangular chip) and directly apply the chip to mass spectrometer.

In a preferable embodiment, the material of the protein chip consists of aluminum plate as a base and silica as an adsorbent material. The aluminum plate is used for transfer by an electric force, and a different conductor is also usable. For transfer by diffusion, an insulator (ceramic, plastic and the like) can be used. As the adsorbent material, silica on which any protein can be transferred is used, but other materials mentioned above can be also used depending on the object.

The protein spread in the gel after migration is transferred onto a protein chip by various methods (diffusion, electric force etc.). This step is generally called blotting. The efficiency of transfer is an important factor in relation to the measurement limit of the subsequent mass spectrometric analysis. In addition, denaturing of protein during migration and blotting should be avoided as far as possible. This is because when a soluble protein binds to a membrane protein, if one of them is denatured or the native three-dimensional structure is not maintained, the binding will not be completed. For this device to be applicable broadly to the study of proteomics, the composition of the molecules that cover the surface of the protein chip is significant. To improve efficiency of blotting greater adsorption capacity of protein, capability of adsorbing any protein (rather than adsorption of certain kind of protein alone) and constant adsorption rate of any protein are required, and to enable binding with a membrane protein, retention of the three-dimensional structure of protein after transfer and adsorption onto a protein chip are essentially required.

The present invention also provides a protein chip wherein the protein spread in the gel after migration as mentioned above is transferred to and located on each small compartment of the protein chip. When the information of molecular weight and the like of the soluble protein located

on each small compartment is obtained in advance by the mass spectrometry to be mentioned below, the membrane protein captured on the protein chip can be analyzed by bringing a membrane protein in contact with the protein chip and applying mass spectrometry.

2. Analysis of protein on protein chip

The protein on a protein chip can be analyzed by directly inserting the protein chip into mass spectrometer. By the "mass spectrometry" is meant a method and a device for the measurement and detection of molecular weight of a substance, which includes ionization of a sample in a gaseous state, casting the ionized molecules and molecule fragments thereof into an electromagnetic field, separating them according to the mass number/charge number based on the migration state, and determining the spectrum of the substance.

The analysis of the ligand fixed on a protein chip according to the present invention can be done using any type of commercially available mass spectrometer, but more preferably done using a mass spectrometer that utilizes; the MALDI-TOFMS method consisting of matrix-assisted laser desorption/ionization (MALDI) wherein a sample is mixed with a matrix that absorbs laser beam and dried to allow crystallization, the crystals are ionized and introduced into vacuum by energy transfer from the matrix and by high-intensity laser pulse, and of time-of-flight mass spectrometry (TOFMS) wherein the mass number is analyzed based on difference in the times of flight of sample molecule ions caused by initial stage acceleration; a method wherein one protein is placed in one liquid drop and is ionized directly and electrically from the liquid; a nano-electrospray mass spectrometry (nano-ESMS) method wherein sample solution is electrically sprayed into the air, and each unfolded protein multivalent ion is led into a gaseous phase, and the like.

The present invention provides a method for detecting

and analyzing a membrane protein together with its ligand by combining the analysis of the ligand on the protein chip by a protein chip on which the above mentioned ligand (soluble protein) and by mass spectrometric analysis with the below-mentioned membrane-protein-transferred liposome and the below-mentioned membrane-protein-ligand complex formation.

3. Separation of membrane protein and transfer to a liposome

This step includes separation of membrane fraction from a cell, preparation of liposome, fusion of membrane fraction and liposome, adjustment of particle size of fused liposome (membrane-protein-transferred liposome), and where necessary, preservation of fused liposome.

For separation of a membrane fraction from a cell, conventional methods can be used. For example, a target cell is obtained and homogenized in a suitable buffer solution in the presence of various protease inhibitors, or suspended in a cell disruption device such as Polytron and the like, or ruptured by a low osmotic pressure shock, or a cell membrane is destroyed by ultrasonication. Thereafter, the cell membrane fractions and organelle membrane fractions are prepared by density gradient centrifugation using various media.

As a method for the preparation of liposome various known methods can be used. Typically but not limitedly, a selected lipid liquid mixture is homogeneously dissolved in an organic solvent, the solvent is completely vaporized in argon gas and hydrated in a buffer solution to generate a liposome. The composition of liposome is important. In general, a cell membrane contains cholesterol abundantly, but lipid bilayer constituting organelle such as endoplasmic reticulum and the like contains a little or no cholesterol. Therefore, the composition of the liposome to receive membrane protein becomes an extremely critical factor in determining which membrane protein of the cell is to be transferred to the liposome. Those of ordinary skill in the art can determine

appropriate lipid constituting the liposome depending on the derivation of the membrane protein.

As a method for fusion of membrane fraction and liposome various known methods can be used. For example, a method including mixing the both in a suitable proportion and then repeating freezing-thawing, a method including placing an aqueous solution containing a membrane fraction on a film made from a liquid mixture of a selected lipid and then transferring the membrane protein to the lipid bilayer by hydration, or a different method can be used for this purpose. Those of ordinary skill in the art can adjust the desired kind and number of membrane proteins to be fused or transferred to a liposome by carefully controlling the mixing ratio of the both. This technique is absolutely important to facilitate the analysis by reducing the noise proteins (noise peaks) in measuring and determining the molecular weights of both the membrane protein and ligand that formed the complex.

For adjustment of particle size of liposome to be fused (membrane-protein-transferring liposome), both the filtration and supersonication can be used in the present invention.

The development of a method for stably preserving a liposome (membrane-protein-embedded liposome) thus obtained comprising the target membrane protein attached to or penetrating a lipid bilayer is extremely important to make the study of proteomics available anywhere any time and at any institutions that do not have mass spectrometer and the like. Several additives developed for preservation of a simple liposome can be used for this end.

The above-mentioned method explains the present method through a manual example. It is needless to say that an automatic method and a semi-automatic method can be used. Automatic addition and removal of reagents, and automatic continuation of steps can minimize the volume of the necessary reagents and more careful control of the reaction conditions

enhances the reproducibility of the results, whereby the method can be applied to the ultimate goal of the present invention.

4. Binding of soluble protein and membrane protein

This step includes binding reaction of a ligand transferred onto a protein chip and a membrane-protein-embedded liposome, removing by washing the liposome non-specifically bound on the protein chip, and removing by lysing the liposome to form a complex of only the ligand and the membrane protein on the protein chip.

As a pretreatment of the binding reaction, a step for coating (blocking) the adsorptive surface of a protein chip with a suitable substance for the purpose of preventing a non-specific adsorption reaction on the protein chip other than physiological ligand-membrane protein interaction can be added. The requirements of a blocking agent are that it can prevent non-specific adsorption of hydrophilic head of the lipid bilayer, it can prevent non-specific adsorption of membrane protein, other than the target membrane protein, which has been transferred to the liposome, it is not a multi-component system that makes subsequent analysis unattainable but a system wherein its components are known and uniform in molecular weight, it consists of molecules that do not absorb ionization energy, and the like.

As the reaction method, simple immersion, shaking and the like may be employed. As a method for increasing the concentration of a membrane-protein-embedded liposome near the protein chip, concentration of liposome by electric force is recommended. Inasmuch as a liposome is negatively charged as a whole, liposome transfers to the surface of a protein chip and concentration near the ligand increases, once cathode is set at the bottom of the protein chip and anode at the upper part of the reaction tank.

The liposome non-specifically bound onto the protein

chip can be removed by washing with a washing buffer having an appropriate salt concentration and composition. While the temperature conditions during washing are important, those of ordinary skill in the art can determine suitable conditions as necessary.

The method for removing the liposome by lysis is exemplified by a method comprising bringing a suitable organic solvent (glycerol, acetonitrile, alcohol, dioxane, DMSO, DMF and the like) in contact with a protein chip after adjusting its concentration as appropriate with the buffer. The use of a mild detergent (e.g., octylglucose and the like) is also effective.

5. Device and program for ligand-membrane protein analysis

In this way, the ligand-membrane protein complex formed on the protein chip can be directly measured by subjecting the chip to the aforementioned mass spectrometer, whereby the molecular information of a ligand and a membrane protein can be simultaneously obtained.

6. Construction and analysis of database

The measurement results of the above-mentioned membrane protein-ligand complex are input to a "ligand-receptor (membrane protein) matrix table" (Fig. 3) previously set and new data are added at any time to construct a database for diagnostic determination.

Taking the method using a protein chip introduced exemplarily in the above as an example, line numbers (1-25) and row symbols (A-Y) are assigned to cover the entire area of the protein chip, thereby to allocate numbers (A1-Y25) to each compartment (4 mm x 4 mm) of total 625 (25 x 25) compartments in one-to-one correspondence (Fig. 3). As a result, the entire ligand transferred onto the protein chip after electrophoresis can be sorted out by the ligand-receptor (membrane protein) matrix compartment numbers. Needless to say, the corresponding receptor obtained thereafter by reaction with a receptor-

embedded liposome can be sorted out by the same compartment numbers, and a substance group that gathers under a certain compartment number are presumed to bind mutually and physiologically.

In one specific embodiment, a target body fluid (considered to contain a soluble ligand) such as serum and the like of a healthy subject and the same kind of target body fluid of a patient having a specific disease are separately applied first to two-dimensional electrophoresis and, after blotting to a protein chip, applied to mass spectrometry and the like for the measurement. At this point, increase and decrease of ligand due to the disease becomes clear and can be entered in the database.

Then, a target body fluid such as serum and the like of a healthy subject is electrophoresed on two sheets of gel followed by blotting in the same manner as above, and two protein chips, on which the ligand of the healthy subject has been transferred, are prepared. The same kind of cells are recovered from the healthy subject and the patient, and respective membrane proteins are transferred to liposomes by the aforementioned membrane protein separation and liposome translocation means to prepare membrane-protein-embedded liposomes. Using a soluble protein-membrane protein binding means, the membrane-protein-embedded liposomes derived from the healthy subject and the patient are individually reacted with respective protein chips, on which the ligand of the healthy subject has been transferred, and a ligand-receptor binding reaction is carried out, which is followed by washing and the like, to obtain a ligand-receptor complex highly purified on the protein chip. The complexes are subjected to measurement by mass spectrometry and the like. At this point, increase and decrease of receptor due to the disease becomes clear and can be entered in the database.

The items of the database are name of disease, name of

body fluid from which ligand is obtained, name of cell from which membrane protein is obtained, as well as ligand-receptor (membrane protein) matrix compartment number, molecular weight or quantification value (or signal intensity output from analysis device such as peak height, peak area and the like) of the results of mass spectrometry, and the like.

The mass spectrometry results are input to the database according to an automating program and the results thereof are expressed in a differential display as shown in Fig. 3 wherein, in the triangle on the right half of "ligand/receptor (membrane protein) matrix table", an increase of ligand is shown with red color, a decrease with blue color and absence of change with yellow color and, likewise in the triangle on the left half, an increase of receptor is shown with red color, a decrease with blue color and absence of change with yellow color. In Fig. 3, patterns are employed instead of display in colors.

Approximate manipulation time of the above is overnight (about 12 hours) for simultaneous electrophoresis of 4 sheets, about 6 hours for simultaneous preparation of 2 kinds of membrane fractions, about 2 hours for simultaneous translocation to liposomes of 2 kinds of samples, about 1 hour for simultaneous ligand-receptor binding reaction of 2 kinds (total time for the above steps about 21 hours), about 52 hours of analysis of 625 compartments by commercially available TOF-mass spectrometer. When one mass spectrometer is used, total steps 21 + 52 x 4 = 229 hours are necessary, and when 4 mass spectrometers are used, 21 + 52 x 1 = 73 hours are necessary. The rate-determining steps are electrophoresis, preparation of membrane fraction and mass spectrometric analysis. When these are improved and automated, the manipulation time can be drastically shortened. For example, the electrophoresis device may be capable of treating 100 sample at a time for 5 hours, preparation of membrane fraction,

transfer to liposome and ligand/receptor binding reaction may be all automated and integrated into one device that may be able to simultaneously treat 20 samples in 3 hours. Furthermore, the analysis time by mass spectrometer may be shortened to one-fifth.

Approximate time necessary for constructing the database for determining diseases after such shortening is assumed by rough calculation, to analyze 1000 cases of each example of 10 disease groups by the above-mentioned method with 10 sets of this device of the invention and 100 sets of mass spectrometer, using serum and one kind of target cell of a disease as a sample, to be 10 x 1000 x 3 x 5 / 99 / 10 (152) hours for electrophoresis, 10 x 1000 x 2 / 19 / 10 (105) hours for preparation of protein chips for mass spectrometric analysis, and 10 x 1000 x 10.4 x 3 / 100 (3120) for mass spectrometric analysis, thus the rate is defined to be 3120 hours (130 days) of mass spectrometric analysis. Therefore, the database for determining the predetermined 10 diseases can be constructed within half a year, after which integrated diagnoses of 10 diseases in one day becomes affordable.

The present invention also provides a tool useful for the development of therapeutic agents of the disease associated with ligand/membrane associated protein. The function analysis of ligand/membrane associated protein occupies an important field of biology (inclusive of medicine and agriculture) in the 21st century, and a larger part of the elucidation of the biological function by molecular biology will be clarified in association with some membrane associated protein. While increase and decrease of ligand and increase and decrease of receptor involved with a specific disease can be clarified from the above-mentioned database for diagnosis (the data up to this point is utilized for determination of diagnosis), a further advantage of the present invention rests in the simultaneous determination of counter molecules of both

the ligand and membrane protein, that means specific ligands and specific membrane proteins that bind physiologically. A novel substance in this area has been found by finding one of them (e.g., a ligand) by accident or for some other purpose and then finding the corresponding receptor using the ligand being labeled, or by finding a pair of ligand and receptor by other way round. Until this point, accidental coincidence and long period of patience is required. There are a number of ligands and membrane proteins having unknown functions, and receptors that bind with artificial substances (medicines) but whose physiological ligands are not identified. When plural molecular species (at least two molecular species including one ligand and one corresponding receptor) are acknowledged in somewhere in the ligand-receptor (membrane protein) matrix compartment number obtained by inputting the measurement results of this method and the existent amount increases or decreases disease-specifically, all molecules located at the compartment number can be provided as disease-associated ligand/membrane proteins, and as highly promising candidates for successful studies of drug discovery. For this purpose, a tandem mass spectrometer is used. This enables presumption of the amino acid sequences of all the molecular species that can be ascribed to a specific compartment number.

Because the present method can analyze any membrane protein of a certain cell membrane, related disease and target cell membrane proteins can be analyzed systematically by one cycle of manipulation, rather than finding each individual membrane protein involved in a disease one by one relying on accidental coincidence.

Moreover, when membrane protein of organelle membrane, such as mitochondrial membrane, is focused on, a drug discovery strategy no one would ever have imagined to date, such as classification of diseases, clarification of relationship between diseases, or development of group-

specific therapeutic agent, can be afforded based on changes in the ATP producing capability.

The present invention is explained in more detail in the following by referring to Examples that are for exemplification only, and do not limit the present invention in any way.

Examples

Example 1 Blotting of urokinase onto protein chip

Urokinase (10, 13, 16, 20, 33 μg) was loaded in the same well of polyacrylamide gel at constant time intervals and electrophoresed. After the completion of the migration, the gel was peeled off from the glass plate and, after substituting the solution in the gel with a blotting buffer (5% acetonitrile/125 mM NaCl/PBS) for 5 min, subjected thermal blotting (diffusion blotting) onto an aluminum plate surface-processed with a hydrophobic material having 16 carbons. As the blotting buffer, used was phosphate buffer containing 5% acetonitrile and 125 mM NaCl and blotting was performed at 35°C for 4 h. Fig. 4 is a photograph showing the protein chip after blotting and the gel before and after blotting stained with coomasie brilliant blue. Urokinase band on the protein chip is not visible to the naked eye, but since the urokinase band on the gel is fainter after blotting, a certain amount of urokinase was suggested to have been transferred onto the protein chip.

Example 2 Identification of urokinase on protein chip by mass spectrometric analysis

After polyacrylamide gel electrophoresis, urokinase transferred onto the protein chip by thermal blotting (diffusion blotting) was directly measured by mass spectrometer, the results of which are shown in Fig. 5. As the conditions of mass spectrometry, Sinapinic acid (saturated solution dissolved in 50% acetonitrile/0.5% trifluoroacetic

acid) was used as an energy absorbing substance, which was added by 0.5 ml/spot and air dried, and then the same amount was added and air dried. The measurement was performed by SELDI ProteinChip® System manufactured by Ciphergen Inc. The mass calibration was external calibration using bata-lactoglobulin A (bovine), Horseradish peroxidase and conalbumin (chicken). The measurement parameters of SELDI ProteinChip® System were; Detector voltage 2200V, Detector Sensitivity 10, Laser Intensity 285. As a result, one peak was observed at the site of molecular weight 49461.2. Since the urokinase used for examination was precursor and the theoretical molecular weight anticipated from the amino acid sequence is 48,525, addition of sugar chain molecule is suggested.

Example 3 Transfer rate of urokinase onto protein chip

Urokinase transferred onto protein chip was quantitated by mass spectrometric method. Using standard urokinase 25, 12.5, 6.25 ng, the standard curve of the peak height obtained by mass spectrometry versus concentration was drawn. Thereafter, urokinase (2,500 ng) was loaded on a polyacrylamide gel and electrophoresed. Urokinase transferred to a protein chip by thermal blotting (diffusion blotting) was directly measured by mass spectrometer. As a result, a peak height corresponding to 25 ng was detected as shown in Fig. 6. From this result, the transfer rate from the gel to the protein chip by thermal blotting method was estimated to be about 1%.

Fig. 1

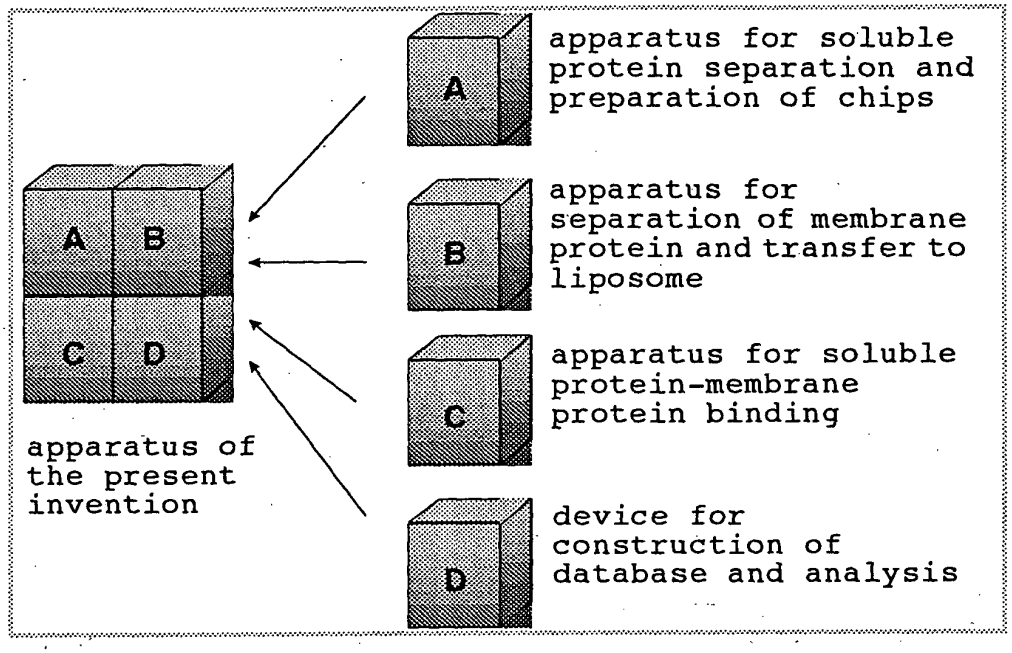

A
B
C
D
apparatus of the present invention
apparatus for soluble protein separation and preparation of chips
apparatus for separation of membrane protein and transfer to liposome
apparatus for soluble protein-membrane protein binding
device for construction of database and analysis

Fig. 2

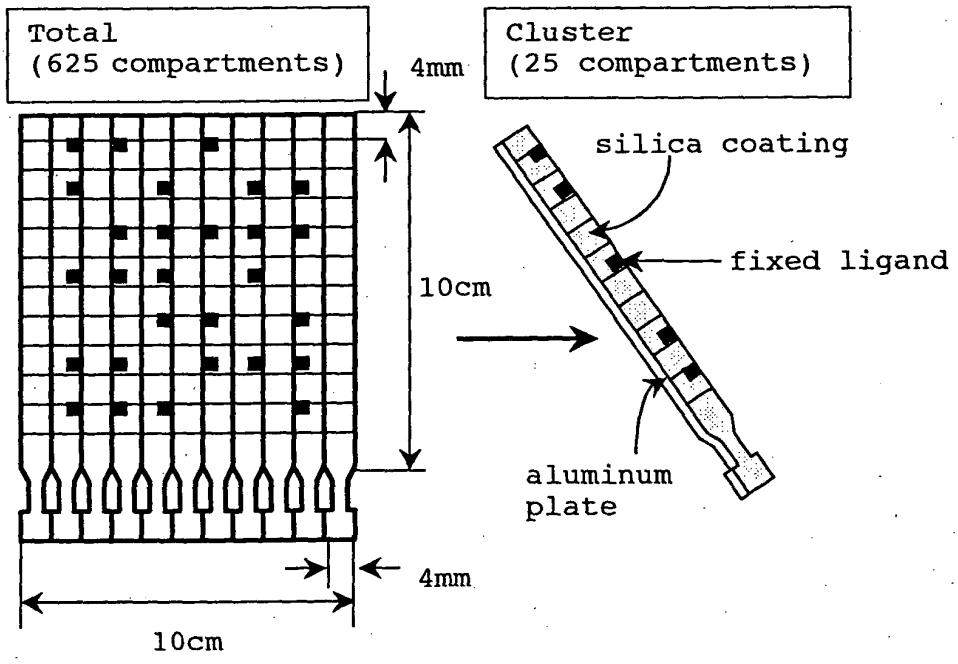

Total
(625 compartments)
4mm
10cm
4mm
10cm
Cluster
(25 compartments)
silica coating
fixed ligand
aluminum plate

Fig. 3

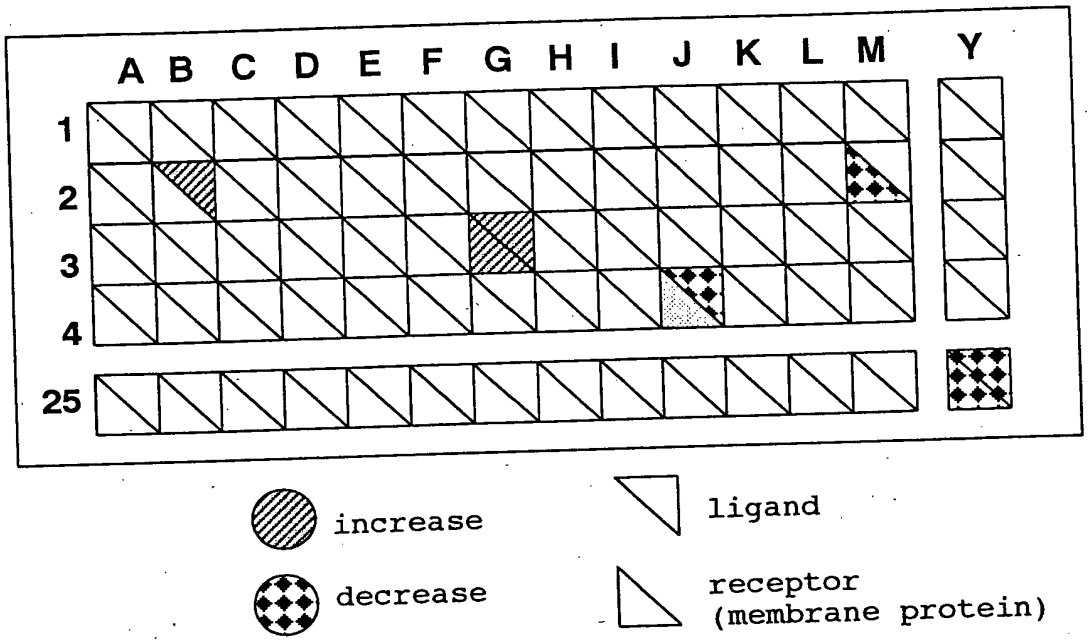

A B C D E F G H I J K L M Y
1
2
3
4
25
increase
decrease
ligand
receptor
(membrane protein)

Fig. 4

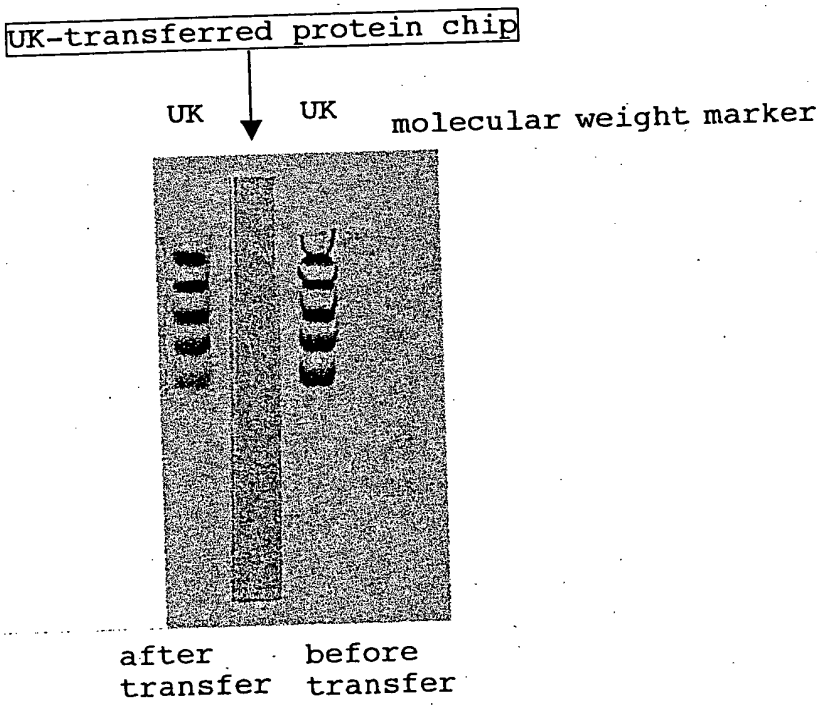

UK-transferred protein chip
UK
UK
molecular weight marker
after transfer
before transfer

Fig. 5

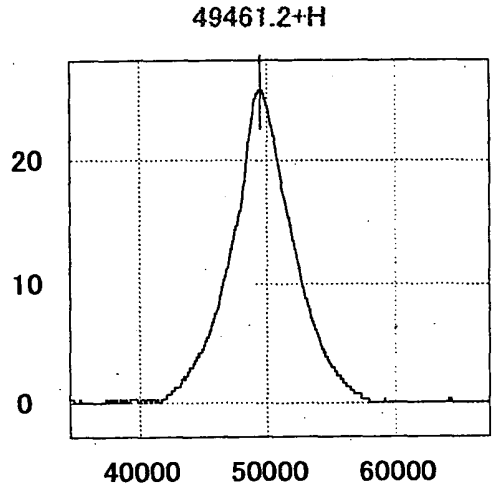
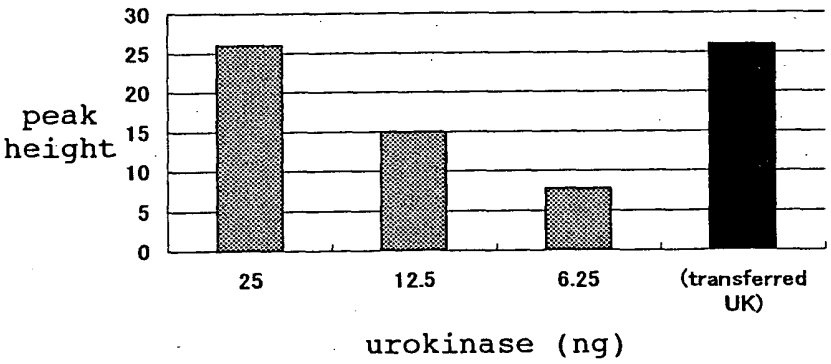

49461.2+H
20
10
0
40000
50000
60000

Fig. 6


peak height
30
25
20
15
10
5
0
25
12.5
6.25
(transferred UK)
urokinase (ng)